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EVALUATION OF CHLOROPENTAFLUOROBENZENE IN A BATTERY OF IN VITRO SHORT TERM ASSAYS

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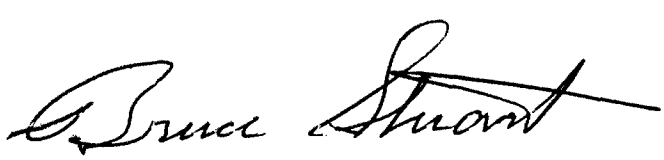

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A fully substituted halogenated benzene, chloropentafluorobenzene, was evaluated in a battery of in vitro short-term bioassays to assess its potential biological activity. The assays conducted were Ames Salmonella/mammalian microsomal mutagenicity assay, the CHO gene mutation assay, the CHO/sister chromatid exchange and chromosome aberrations assay, a DNA repair assay utilizing primary rat hepatocytes and the BALB/c-3T3 cell transformation assay The test compound, although a liquid, precipitated out of solution in solvents compatible with the bioassays. Therefore, the actual soluble concentrations in the test medium were not known. Under these conditions, chloropentafluorobenzene produced a positive response in the CHO/chromosome aberrations assay and in the primary rat hepatocyte/DNA repair assay but was not active in the remaining four in vitro assays.

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PREFACE

This is the final report of work performed by Arthur D. Little, Inc., for the United States Air Force under Work Order 0012, Contract F33615-81-D-0508, Work Unit 63020155, "Mutagenic, Teratogenic, and Carcinogenic Potential of Air Force Chemicals." This report describes accomplishments from July 1, 1985, to November 1, 1985. Andrew Sivak, Ph.D., was Program Manager for the program. Alice S. Tu, Ph.D., was Task Manager for this Work Order. Key personnel involved with this project included: Mildred G. Broome, Ph.D., Patricia A. Breen, B.S., Wendy C. Hallowell, B.S., Kathleen M.B. Findlen, B.S., Karen M. Hatch, B.S., and Stacie L. Pallotta, B.S. Marilyn George, Biochemical Toxicology Branch, Toxic Hazards Division, Harry G. Armstrong Aerospace Medical Research Laboratory, was technical monitor for the Air Force.

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LIST OF ABBREVIATIONS

AFB_1	aflatoxin B_1
BrdU	bromodeoxyuridine
CA	chromosome aberrations
CHO	Chinese hamster ovary
CPFB	chloropentafluorobenzene
CPP	cyclophosphamide
DFCS	dialyzed fraction of fetal calf serum
DMN	dimethylnitrosamine
DMSO	dimethylsulfoxide
EDTA	disodium ethylenediamine-tetraacetate
EGTA	ethyleneglycolbis (β-aminoethylether)N,N'-tetraacetic acid
EMS	ethylmethanesulfonate
EtOH	ethanol
FCS	fetal calf serum
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
KCl	potassium chloride
MCA	3-methylcholanthrene
NADP	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
PBS	phosphate-buffered saline
S9	9000 xg supernatant
SCE	sister chromatid exchange
TG	thioguanine
UDS	unscheduled DNA synthesis

INTRODUCTION

This is a report of the evaluation of chloropentafluorobenzene in a battery of in vitro short-term bioassays to assess the potential biological activity of this halogenated benzene. The assays conducted included the Salmonella/mammalian microsomal mutagenicity assay (Ames assay), a mammalian specific locus gene mutation assay (CHO/HGPRT assay), two cytogenetic assays measuring sister chromatid exchange (CHO/SCE assay) and chromosome aberrations (CHO/CA assay), a DNA repair assay using primary rat hepatocytes (rat hepatocyte/UDS assay), and a mammalian cell transformation assay (BALB/c-3T3 assay). All these assays are well-validated systems and are representative of varied endpoints designed to detect genotoxic agents which may have different mechanisms of action.

The Ames mutagenicity assay measures the ability of chemical agents to induce mutations in certain strains of bacteria. The suspect chemicals are tested with five specially constructed mutants of Salmonella typhimurium, selected for sensitivity and specificity in being reverted from a histidine requirement back to prototrophy by chemical mutagens. By adding homogenate of rat liver (S9) to the plates, thus incorporating an aspect of mammalian metabolism in vitro, the assay may also detect potential mutagens which require metabolic activation.

The CHO/HGPRT assay measures the ability of a test agent to induce forward mutations at the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of Chinese hamster ovary cells on the basis that presumptive mutants defective in the enzyme HGPRT are unable to convert purine analogues such as 6-thioguanine to toxic metabolites. Hence, in a selection medium containing 6-thioguanine, the mutant cell will be able to grow, while the wild type cells are killed. To enhance detection of compounds which require metabolic activation to be mutagenic, the assay is conducted in the absence and presence of an Aroclor 1254-induced rat liver microsomal (S9) fraction.

The assay for sister chromatid exchange (SCE) in Chinese hamster ovary (CHO) cells measures the ability of an agent to increase SCEs above an established baseline. SCEs are detected from the differential staining of chromatids in cytological preparations of metaphase chromosomes. These changes presumably involve DNA breakage and reunion and are therefore thought to be indications of DNA damage.

Detection of chromosome aberrations (CA) is a classical method of assessing the effect of physical and chemical agents on the genetic apparatus of cells. Alterations in chromosomes of CHO cells as a result of exposure to a test agent are visualized in stained cytological preparations of metaphase chromosomes and are categorized as chromosome aberrations, chromatid aberrations, or numerical aberrations. The CHO/CA assay is generally carried out in the absence and presence of an Aroclor 1254-induced rat liver S9 fraction.

The primary rat hepatocyte unscheduled DNA synthesis (UDS) assay measures the excision repair of DNA lesions induced by genotoxic agents. The UDS is determined by autoradiographic detection of the amount of ^{3}H-thymidine that has been incorporated into the nuclear DNA as the result of treatment with test agents.

The BALB/c-3T3 cell transformation assay is designed to measure the ability of chemical agents to induce alterations in a population of cells (derived from mouse embryo fibroblasts) from a pattern of controlled monolayer growth to one exhibiting foci of disoriented, piled up growth against the background monolayer cells. In vitro transformation is based on morphological events that mimic oncogenesis in vivo. Transformed cell populations in general assume other properties of tumor cells such as growth in semi-solid medium, and often, tumorigenicity in syngeneic, immuno-suppressed host animals.

MATERIALS AND METHODS

TEST COMPOUNDS

Two 25-gram bottles of chloropentafluorobenzene (lot # JK3511TH), a clear liquid of 95% purity from Aldrich Chemical Company, were provided by the U.S. Air Force. The test sample was stored at room temperature. The positive control ethylmethanesulfonate (EMS) was purchased from Eastman Kodak Company; dimethylnitrosamine (DMN), 9-aminoacridine and sodium azide were obtained from Sigma Chemical Company; 2-aminoanthracene and 2-nitrofluorene were from Aldrich Chemical Company; 3-methylcholanthrene and cyclophosphamide were provided by Radian Corporation and the National Cancer Institute, respectively. These control compounds are stored in a -20°C freezer or a 4°C refrigerator designated for hazardous substances. All compounds were used as received without further chemical analysis.

SALMONELLA/MAMMALIAN-MICROSOMAL MUTAGENICITY ASSAY (AMES)

The Salmonella typhimurium strains used in this study were obtained from Dr. Bruce Ames, University of California, Berkeley, California, and are identified as TA-98, TA-1538, TA-100, TA-1535, and TA-1537. Their properties and specific details of the assay have been described by Ames and co-workers (Mut. Res. 31:347, 1975). Master cultures from which working cultures are prepared are maintained frozen in liquid nitrogen. Working cultures are maintained at -80°C. Confirmation of strain performance is conducted every six months.

Ames Assay Test Procedure

The Ames assay was conducted according to our standard operating procedure #CB/M-812c. After a preliminary toxicity assay, chloropentafluorobenzene was assayed in the standard plate incorporation assay with and without metabolic activation (Aroclor 1254 induced rat liver microsomal fraction, S9). The test organism (0.1 ml), the appropriate sample dilution (0.1 ml) and the S9 mixture (0.5 ml, if required) were added to 2.0 ml of 0.6% molten top agar containing the histidine/biotin supplement. The molten top agar mixture (in duplicate) was mixed by

vortexing and poured on minimal glucose agar plates. The solvent control was the highest volume of DMSO used in each experiment. Positive controls are listed below and included compounds which do and do not require metabolic activation.

Tester Strain	Positive Control Chemicals
All Strains	2-Aminoanthracene, 10 µg/plate
TA-98, TA-1538	2-Nitrofluorene, 10 µg/plate
TA-100, TA-1535	Sodium azide, 10 µg/plate
TA-1537	9-Aminoacridine, 50 µg/plate

After incubation for 48 hours at 37°C, mutant colonies were counted and results reported as (average) total number of revertants per plate ± the standard deviation.

Quantitation of Data

The mean number of revertants and the standard deviation were calculated for each condition and the results expressed as mean number of revertants per plate.

Acceptability of Assays and Criteria for Response

The criteria used to determine the validity and results of an assay include the following:

- Solvent control values must be within the normal range.
- Number of revertants induced by positive control chemicals must be within the historical normal range.
- There must be a healthy (background) "lawn" of cells indicating that the test chemicals have not been assayed at concentrations that are so cytotoxic that all induced mutants have been killed.
- Concentrations of materials tested may include toxic doses since mutagenicity and toxicity are related, but a non-toxic dose must also be tested.
- A two-fold increase in numbers of revertants over the spontaneous number of revertants with a positive dose-response relationship is considered a positive test.
- Dose response curves should be reproducible.
- A positive dose-response relationship which does not double the number of spontaneous revertants is an indication of an inconclusive (±) test.

CELL CULTURES

The cells used in the mutagenesis and cytogenetic studies were subclone BH_4 of strain K_1 of the Chinese hamster ovary (CHO) cell line. The stock cultures were originally obtained from Dr. Abraham Hsie's Laboratory (Oak Ridge National Laboratory, Oak Ridge, Tennessee) in April, 1982, and stored in liquid nitrogen.

The CHO cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 µg/ml streptomycin. They were grown in monolayer culture at 37°C in an atmosphere of 5% CO_2 and 95% humidity. For subculture, the cells were detached with a 0.05% trypsin/0.02% EDTA solution. Under these culture conditions, the cells have a doubling time of approximately 12 hours and maintain a stable karyotype of 19-20 chromosomes.

Primary rat hepatocytes were isolated by a two-step liver perfusion procedure as generally described by Williams (Cancer Letts 1:231, 1976). The livers of adult male Fischer rats (150-200 gm) were perfused in situ with 0.5 mM ethyleneglycol-bis-(β-aminoethylether)N,N'-tetraacetic acid in Hank's balanced salt solution buffered with 0.05 M N-2-hydroxy-ethylpiperazine-N'2-ethanesulfonic acid (HEPES), pH 7.4 for 4 min at 40 ml/min followed by a second perfusion of a collagenase (100 units/ml) solution in Williams medium with HEPES for 10 min at 20 ml/min. The hepatocytes were detached by gently combing the liver with a stainless steel comb. The cells were sedimented at 50 xg for 4 min.

The cells used in the in vitro transformation assay were BALB/c-3T3 cells from a mouse fibroblast cell line. The original cell stock of BALB/c-3T3 clone 1-13 cells were obtained from Dr. T. Kakunaga, National Cancer Institute, in January, 1977. These cells were expanded in culture and stored in liquid nitrogen in ampules at 10^6 cells/ampule. Each working stock of target cells, consisting of 50-60 ampules of cells (k series), was expanded from a frozen ampule of the original stock cells from Dr. T. Kakunaga. The medium used in the assay was Eagle's minimal essential medium supplemented with 10% fetal calf serum; penicillin and streptomycin were employed at 50 units/ml and 50 µg/ml, respectively. The cells were grown in a 5% CO_2 incubator at 37°C and 95% relative humidity.

METABOLIC ACTIVATION SYSTEM

A complete activation system consisted of F12 medium buffered with 0.02M HEPES, pH 7.4, $MgCl_2$(10 mM), glucose-6-PO_4 (5mM), 1 mM each of NADP and NADH, 0.4 mM NADPH and S9 (Aroclor 1254 induced Sprague-Dawley rat liver microsomal fraction prepared according to Ames et al., and our SOP #CB/M-526). The final concentration of S9 used was 2 mg/ml and 0.4 mg/ml in the CHO/HGPRT assay and in the two CHO/cytogenetic assays, respectively. The S9 mix was prepared fresh and added to the cells immediately prior to the addition of test chemical.

CHO/HGPRT GENE MUTATION ASSAY

Initial Cytotoxicity Assay

The cytotoxic effect of chloropentafluorobenzene on CHO cells was determined by a clonal assay which measured the reduction in colony forming ability of CHO cells. The CHO cells were plated at 200 cells/60 mm dish 24 hours prior to chemical treatment. After a 16 hour exposure period to the test sample (5 hours in the presence of S9 metabolic activation), the medium containing the test sample was removed, the cells were rinsed with phosphate buffered saline (PBS) and fresh medium added. The cells were incubated in a 5% CO_2 incubator at 37°C for 7 days to allow colonies to develop. At the end of the incubation period, the culture dishes were rinsed with PBS, fixed in 100% ethanol, stained with 5% Giemsa and the colonies were counted. The cloning efficiency and the surviving fraction were calculated from the clonal counts obtained in the control and treated conditions. The concentrations selected for the mutation assay were based on results of this clonal assay.

Parallel Cytotoxicity Assay

In parallel with the mutation assay, CHO cells were plated at 200 cells/60 mm dish and treated with the test sample at the same time as the mutation dishes to determine the cytotoxic effect of the test sample at concentrations tested for the mutation assay. The clonal assay procedure was as described for the initial cytotoxicity assay.

CHO/HGPRT Mutation Assay Procedure

Mutation induction at the HGPRT locus was measured as described in our standard operating procedure #CB/M-806b. Briefly, cells were plated at $5x10^5$/100 mm dish in Ham's F12 medium containing 5% dialyzed fetal calf serum (DFCS). After 24 hours, duplicate cultures were treated with the test sample. The solvent control was the highest concentration of DMSO used in the experiment and the positive control was ethylmethanesulfonate (EMS) at 248 µg/ml in the absence of S9 or dimethylnitrosamine (DMN) at 500 µg/ml in the presence of S9. The cells were treated for 16 hours (5 hours in the presence of S9) after which the medium was removed, the cells were rinsed with PBS and fresh culture medium containing 5% DFCS was added. After approximately 24 hours of incubation, the cells from each dish were trypsinized, counted and replated in duplicate at $1x10^6$ cells/100 mm dish in 10 ml of medium containing 5% DFCS. The cells were subcultured once every 2-3 days to allow a total expression period of 7-9 days. To select for thioguanine-resistant mutants, the cells were trypsinized, counted and replated at $2x10^5$ cells/100 mm dish in 10 ml of hypoxanthine-free growth medium containing 5% DFCS and 10 µM 6-thioguanine (TG). Ten dishes (5 from each duplicate for a total of $2x10^6$ cells screened) were used per test condition. From the same stock cells, five replicates were plated with 200 cells/60 mm in medium without TG to determine cloning efficiency. The cultures were incubated for 6-9 days, the dishes were then rinsed with PBS, fixed in methanol, stained with 5% Giemsa and the colonies counted.

Data Quantitation

The mutation frequency was calculated by dividing the total number of TG-resistant colonies scored by the number of cells plated ($2x10^6$) corrected for the cloning efficiency and expressed as mutants per 10^6 clonable cells.

Acceptability of Assay and Criteria for Response

A mutation assay is considered acceptable if it meets the following criteria:

- the cloning efficiency of the CHO cells is ≧60%,
- three of the four test concentrations have an acceptable number of cells ($5x10^5$) analyzed for HGPRT mutation,
- the positive control (EMS or DMN) induces a statistically significant (Student's t-test, $p<0.01$) mutation frequency above the untreated or solvent control.

A test chemical is considered positive in the mutation assay if:

- it induces a statistically significant (Student's t-test, $p<0.01$) mutation frequency above the control in a dose-dependent manner; and
- a net increase in mutant colonies of treated above the control is observed in at least two of the concentrations tested.

These criteria are not absolute and other extenuating factors may enter into the final interpretation of results.

CYTOGENETIC ASSAYS

Dose Selection

The selection of concentrations for the cytogenetic assays (SCE and CA) was based partially on the results of the clonal cytotoxicity assay conducted for the CHO/HGPRT assay. Neither a toxicity measure by the reduction in clonal growth nor the decrease of cell numbers in mass culture predict well the concentrations at which cytogenetic results could be obtained. Therefore, part of the cytotoxicity assessment was incorporated into the actual assay. Cultures were exposed to eight concentrations of the test sample in half-log dilution, the highest concentration being one in which the DMSO solvent concentration did not exceed 1% or that which produced no growth in the clonal assay. Four of the eight concentrations, based on analyzable metaphases and spaced among concentrations which provided a dose-response relationship, if possible, were selected for scoring.

Sister Chromatid Exchange (SCE) Assay Procedure

Sister chromatid exchanges were examined in CHO cells using our standard operating procedure #CB/M-804. Cells were plated at $3x10^5$cells/100 mm dish containing 10 ml of Ham's F-12 medium with 10% FCS. The following day, cells were treated with the test sample for 24 hours in the absence of metabolic activation. At the time of treatment, bromodeoxyuridine (BrdU) at a final concentration of 10 μM was also added to the cultures. In the presence of S9, the test sample was removed after 2 hours and the cells incubated for an additional 22 hours in medium without test sample but with BrdU. After the addition of BrdU, the cells were protected from light and incubated at 37°C.

Twenty-two hours after the initiation of treatment with the test sample and BrdU, Colcemid at a final concentration of 0.45 μg/ml was added for the last two hours of culture. The cells were then harvested by trypsinization and combined with the culture medium which could contain dividing cells. The cell suspensions were centrifuged and the cell pellets were resuspended in 0.075 M KCl for 7-10 minutes, centrifuged, and fixed in 2:1 methanol:glacial acetic acid. The fixative was changed twice before slides were made by dropping the suspension on clean slides and air-drying. The slides were stained by the fluorescence-plus-Giemsa technique (standard operating procedure #CB/M-515).

A total of 50 metaphases were scored microscopically for each experimental condition (25 from each of the duplicate cultures), and chromosome counts were made on each metaphase scored. The mitotic index was determined by counting 1000 randomly selected cells from each experimental condition and the number of cells which were undergoing mitosis was expressed as percentage of the total cells counted.

Quantitation and Statistical Analysis of Data

The mean number of SCE, standard deviation, and standard error of the mean were calculated for each condition and the results were expressed as the number of SCE per cell. To determine statistical significance of the treated condition above the control, a two-tailed t-test like that used in the HGPRT assay was carried out.

Acceptability of Assay and Criteria for Response

The following criteria were used to evaluate the assay results:

- A minimum of 30 metaphases must be scored for each test condition in at least two of the four doses tested,
- The controls (negative and positive) must have an acceptable number (30 or more) of metaphases scored,
- The positive control must induce a statistically significant ($p<0.01$) increase in SCE above the untreated or solvent control, whichever has the higher frequency,

- An agent is considered positive in the assay if it induces a statistically significant ($p<0.01$) SCE frequency above the control (or solvent control) and responds in a dose-dependent manner, with at least one concentration inducing a 20% increase in SCE above the negative control.

CHO/CHROMOSOME ABERRATIONS ASSAY

CHO/CA Assay Procedure

Exponentially growing CHO cells were plated at $3x10^5$ cells/100 mm dish and incubated for 24 hours. Duplicate cultures were treated with the chloropentafluorobenzene for 24 hours in the absence of S9. In the presence of S9, the medium with test sample was removed after 4 hours and incubated for an additional 20 hours in medium without test sample. Two hours prior to the collection of cells, Colcemid was added to the culture at a final concentration of 0.45 µg/ml. At the end of the incubation period, the cells were collected by trypsinization, swelled in hypotonic medium (0.075 M KCl), and fixed in methanol: glacial acetic acid (2:1). The fixed cells were dropped on clean slides, stained in a 4% Giemsa solution, pH 6.8, rinsed in water, and air dried. The mitotic index for each test condition was determined by counting the number of cells in mitosis in 1000 randomly selected cells on the slide. Chromosome aberrations were scored microscopically in 100 metaphases for each test condition, 50 from each duplicate. The following categories of aberrations were scored and recorded:

Chromosome Aberrations - changes in the configurations of whole chromosomes observed at homologous sites of both chromatids. These include markers (acentrics, dicentrics, rings, translocations), breaks, fragments and gaps.

Chromatid Aberrations - changes involving individual chromatids of a chromosome. These include interchanges (quadraradials, triradials), breaks, fragments and gaps.

Numerical Aberrations - changes involving many or all chromosomes within a cell. These are scored as pulverized chromosomes or cells with greater than 10 aberrations.

Data Quantitation

Chromosome and chromatid gaps were scored but not included as aberrations in the quantitation of data. For each test condition, the mean number of aberrations per cell ± the standard deviation and the standard error of the mean (S.E.M.) were calculated. Also, the percentage of cells with aberrations was determined from total metaphases scored. Cells with numerical aberrations were excluded in the calculation of aberrations per cell but included in the calculation of cells with aberrations.

Acceptability of Assay and Criteria for Response

An assay is acceptable if:

- a minimum of 60 metaphases are scored for the test condition to be included in the calculation,
- two of the four concentrations tested have acceptable numbers of metaphases scored,
- the positive control (EMS or CPP) induces a statistically significant (Student's t test, $p<0.01$) increase in aberrations over the control.

A test chemical is considered positive in the assay if it induced a statistically significant (Student's t-test, $p<0.01$) increase in aberration frequency above the control in more than one of the concentrations tested. A dose-dependent effect is corroborative evidence of a positive response. The following guideline may also be used in the judgement of a positive response in this assay.

Response	% Cells with Aberrations
-	<4.9
±	5.0 - 9.9
+	10.0 - 19.9
++	20.0 - 49.9
+++	> 50.0

PRIMARY RAT HEPATOCYTE/UDS ASSAY

Dose selection

A preliminary cytotoxicity assay to determine the cytotoxic effect of the test samples on primary rat hepatocytes was not conducted. Rather, the assessment of toxicity was incorporated into the actual assay. Five concentrations of the test sample, in log-fold dilutions, were used. The highest concentration was that which resulted in a DMSO solvent concentration of 0.5%.

Primary rat hepatocyte/UDS assay procedure

Freshly isolated rat hepatocytes were plated at $3x10^5$ viable cells (viability determined by trypan blue dye exclusion) into 35 mm culture dishes containing 22 mm round Thermanox plastic coverslips. The hepatocytes were allowed to attach for 1.5 hours in a humidified CO_2-incubator. The medium was then replaced with fresh medium containing the test sample and 10 µC/ml ^{3}H-thymidine. After incubation for 18 hours at 37°C in a humidified 5% CO_2-incubator, the test medium was removed, the cells were rinsed in PBS, swelled in 1% sodium citrate

and fixed in ethanol:glacial acetic acid (3:1). The coverslips were dried and attached with mounting medium, cell side up, to microscope slides. The slides were dipped in NTB photographic emulsion, dried and exposed in the dark for 10-14 days at 4°C. The slides were then developed for 5 min in D19 developer (Kodak), fixed and stained, air dried and coverslipped in Permount.

Unscheduled DNA "repair" synthesis was determined from nuclear and background silver grain counts made on an Artek (model 880) automatic counter with microscopic attachment. Grain counts over the nucleus and three cytoplasmic areas of the same size were made. At least 50 cells were counted for each test condition.

Data Quantitation

The net nuclear grain (NG) count of UDS for each cell was calculated by subtracting the mean of the three cytoplasmic background counts from the nuclear count. The mean net nuclear grains per nucleus for the total number of cells counted for each test condition and the percentage of cells with NG ≥ 5 was also calculated.

Acceptability of Assay and Criteria for Response

A UDS assay is considered acceptable if it meets the following criteria:

- the viability of attached hepatocytes ≥ 60%,
- at least three concentrations have acceptable numbers of cells (50) counted,
- the positive control (aflatoxin B_1 or DMN) induces ≥ 5 net nuclear count.

A test sample is positive in the UDS assay if:

- it induces a concentration related statistically significant ($p \leq 0.01$) increase in net nuclear counts over the negative control,
- it induces a concentration related increase above the negative control in the percentage of cells with ≥ 5 NG.

These criteria are not absolutes; other extenuating factors may enter into the final evaluation and interpretation of results.

BALB/c-3T3 CELL TRANSFORMATION ASSAY

Initial Cytotoxicity Assay

The effect of the test sample on the survival of BALB/c-3T3 cells was determined by a reduction of cell number in mass culture after

treatment with the test chemical. Exponentially growing BALB/c-3T3 cells were plated at $5x10^3$cells/35 mm well of cluster dishes. After 24 hours, duplicate cultures were treated with varying concentrations of the test sample for 3 days, the exposure time for the transformation assay. At the end of the treatment period, the cells were trypsinized and counted. The surviving fraction of cells treated with the test sample was calculated by comparing them to survival of untreated cells which was considered 100%. The highest test concentration used in the transformation assay was that resulting in 10-20% cell survival.

Parallel Cytotoxicity Assay

The cytotoxicity of the test sample at concentrations tested in the transformation assay was determined as follows: At the end of the three day treatment period, two plates selected from each experimental set were rinsed with PBS, the cells trypsinized and counted. An aliquot of cells from each test condition was replated at 100 cells/plate (5 plates per set) to determine the cloning efficiency of the cells.

BALB/c-3T3 Transformation Assay Procedure

Dishes for transformation assay were plated with cells expanded from frozen stock at 10^4 cells per 60 mm plate. At least 22 dishes were set up for each test condition. Twenty-four hours later, the test samples were added to the appropriate plates. After a three-day treatment, the medium was removed, the plates were replenished with fresh medium and incubated for a total of approximately four weeks. The medium was changed every 4-7 days during the incubation period. At the end of the incubation period, the plates were fixed with methanol and stained with 2-3% Giemsa. Each stained plate was examined for foci under a dissecting microscope. Foci are dense areas of cells over the background monolayer. Foci are classified into 3 types as described by Reznikoff et al. (Cancer Res. 33:3239, 1973). Only Type III foci (aggregations of densely stained cells that are randomly oriented and exhibiting criss-cross array at the edge of the focus) are scored.

Data Quantitation

The mean number of Type III foci per plate and the standard error of the mean as well as the fraction of plates with Type III foci for each experimental set were calculated. To determine if the number of foci/plate of the treated sets is significant ($P<0.05$) above that of the control, a modified t-test is carried out.

$\bar{X}_C$ and $\bar{X}_T$ = mean foci/plate of the control and treated sets, respectively; SE_C and SE_T = standard error of the control and treated sets; and n_C and n_T = number acceptable control and treated plates.

$$t = \frac{\left|\bar{X}_C - \bar{X}_T\right|}{\sqrt{(SE_C)^2 + (SE_T)^2}}$$

$$DF = \frac{\left|SE_C^2 + SE_T^2\right|^2}{\frac{SE_C^4}{n_C} + \frac{SE_T^4}{n_T}}$$

Acceptability of Assay and Criteria for Response

An assay is acceptable if it meets the following criteria:

- A minimum of 10 plates must be scored for each experimental set to be included in the calculation,
- Three of the four test concentrations have the minimum number of acceptable plates,
- The upper limit of Type III foci/plate for the untreated control is 0.75. The lower limit of Type III foci/plate for the positive control (MCA, 2 μg/ml) is 1.20.

A test chemical is considered positive in the assay if it produces a statistically significant ($P<0.05$) increase in Type III foci/plate above the untreated control in at least two of the concentrations tested. The fraction of plates with foci and a positive dose-response are corroborative data used in the final judgement of a positive response.

RESULTS

SOLUBILITY OF TEST CHEMICAL

The solubility of chloropentafluorobenzene (CPFB) was screened in a number of solvents compatible with the in vitro short term bioassays. At a concentration (160 mg/ml) which was commonly used as the primary stock solution, CPFB was not soluble in any of the solvents tested which included dimethylsulfoxide (DMSO), ethanol (EtOH), phosphate-buffered saline (PBS), fetal calf serum and culture medium (Table 1). Since a

relatively homogeneous suspension was obtained with CPFB dissolved in DMSO, it was used as the primary solvent in the various assays. Because of the solubility problem, we did not know the actual dissolved concentration of CPFB in the medium under the conditions in which CPFB was tested.

AMES ASSAY

Preliminary cytotoxicity data with TA-100 in the absence of metabolic activation (Table 2) indicated that chloropentafluorobenzene was toxic at a concentration of 1.6 µl/plate,, causing destruction of the background lawn. Concentrations of 0.32 and 0.064 µl/plate reduced the number of spontaneous revertants compared to the DMSO control, but did not affect the appearance of the background lawn.

A mutagenicity assay with all five tester strains with and without metabolic activation gave no evidence of mutagenicity for chloropentafluorobenzene (Table 3). At 1.6 µl/plate, there was toxicity to all tester strains with and without metabolic activation. At 0.32 µl/plate the chemical was toxic only to TA-98 in the absence of metabolic activation. Positive and negative controls were acceptable and within the normal historical range.

CHO/HGPRT MUTATION ASSAY

The cytotoxicity of CPFB on the CHO cells in the absence and presence of an exogenous metabolic activation system was determined by a clonal survival assay. The results (Table 4) showed that CPFB was cytotoxic only at concentrations (100-300 µg/ml) that were visibly insoluble.

For the CHO/HGPRT gene mutation assay conducted in the absence of exogenous metabolic activation, two separate primary stock solutions of CPFB, 25 mg/ml and 125 mg/ml, were made. The more concentrated stock solution was used for concentrations higher than 100 µg/ml to maintain an acceptable DMSO solvent concentration in the test medium. The results (Table 5) of the cytotoxicity assay which was conducted in parallel with the mutation assay, showed a toxicity profile reflective of the fact that the chemical was not soluble. For example, the cytotoxic effect of CPFB was the same at 100 µg/ml and 500 µg/ml, producing a relative surviving fraction of 0.26-0.24 when two separate stock solutions were used. This suggests that the actual soluble concentration of CPFB at 500 µg/ml, which was derived from the 125 mg/ml stock, was likely no higher than that at 100 µg/ml from the 25 mg/ml stock.

The mutagenicity data (Table 5) showed that CPFB, at two concentrations (50 and 250 µg/ml), produced a mutation frequency higher than the control value. However, since the frequency was quite low and since no consistent dose-dependent effect was observed, this sporadic increase in frequency was not considered a true mutagenic response.

In the presence of an Aroclor-induced rat liver S9 fraction, CPFB also produced no dose-dependent mutagenic effect (Table 6). Since the mutation frequency of the negative controls (medium and DMSO) was higher than generally observed, the assay with metabolic activation was repeated. The results of the repeat assay (Table 7) confirm the earlier negative response.

CHO/SCE ASSAY

The ability of CPFB to induce sister chromatid exchanges in the CHO cells was determined both in the absence and presence of an exogenous metabolic activation system. The results are shown in Tables 8 and 9, respectively. In the absence of a microsomal S9 fraction (Table 8), CPFB produced a statistically significant ($p<0.01$) increase at 300 µg/ml, the highest concentration tested. The increase in SCE, however, was less than 20% above the control, the level of increase that has been judged necessary to conclude a positive response. No statistically significant increase in SCE was observed in the CHO cells exposed to CPFB in the presence of an S9 fraction (Table 9).

CHO/CA ASSAY

In contrast to the results obtained with the SCE assay, CPFB induced chromosome aberrations in the CHO cells in a dose-dependent manner both in the absence (Table 10) and presence (Table 11) of an S9 metabolic activation system at concentrations between 10-300 µg/ml, the same concentrations that were tested in the SCE assay.

RAT HEPATOCYTE/UDS ASSAY

The ability of CPFB to induce unscheduled DNA repair synthesis was determined in the primary rat hepatocyte/UDS assay. The results are shown in Tables 12 and 13. The net nuclear grain counts and the % nuclei with $\geq$ 5 nuclear grains of the negative control were unusually high in the first experiment (Table 12). The reason for this phenomenon is unclear, but may in part be due to variability of animals from which primary hepatocytes were obtained. The results of a repeat assay (Table 13) show that CPFB induced DNA repair synthesis in the hepatocytes.

BALB/c-3T3 TRANSFORMATION ASSAY

Table 14 shows the results of an initial cytotoxicity screen of CPFB on the BALB/c-3T3 cells. Similar to its toxic effect on the CHO cells, CPFB was not substantially toxic on the BALB/c-3T3 cells except at concentrations (100 µg/ml and above) which were visibly insoluble. The results of the transformation assay in Table 15 show that CPFB did not exhibit any transforming activity under the conditions of the assay. At the two highest concentrations (100 and 250 µg/ml), there was evidence that CPFB dissolved the culture dish plastic at localized areas, suggesting an uneven distribution of CBFB under insoluble conditions.

CONCLUSION

The responses produced by CPFB in the six in vitro short term bioassays are summarized in Table 16. The halogenated compound CPFB produced chromosome aberrations in the CHO cells and induced DNA repair synthesis in the primary rat hepatocytes, but had no detectable activity in the remaining four assays. Since CPFB was not soluble under the conditions tested, the actual soluble concentrations in the test medium were not known.

TABLE 1

SOLUBILITY BEHAVIOR OF CHLOROPENTAFLUOROBENZENE
IN VARIOUS SOLVENTS

Solvent	Concentration	Observation
DMSO	160 mg/ml	Form suspension of white precipitates
EtOH	160 mg/ml	Form large white solid mass
PBS	160 mg/ml	Immiscible; form large bead at bottom of tube
Serum[a]	160 mg/ml	Form large bead at bottom of tube
Culture[b]	160 mg/ml	Form white precipitates not as readily suspended medium as in DMSO

[a] fetal calf serum.
[b] culture medium = F12 + 5% DFCS

TABLE 2

CYTOTOXICITY OF CHLOROPENTAFLUOROBENZENE ON
SALMONELLA TYPHIMURIUM STRAIN TA-100

Compound: Chloropentafluorobenzene

Date: July 17, 1985

Organism: TA 100 without S-9

Concentration (µl/plate)	Mean number of Revertants/Plate ± Standard Deviation
200	74±11[a,b]
40	73±1[a]
8	63±2[a]
1.6	64±14[a]
0.32	86±2
0.064	96±6
DMSO Control	
100	137±18

[a] Toxic effect on background lawn noted.

[b] Compound separated out in agar overlay.

TABLE 3

AMES ASSAY - CHLOROPENTAFLUOROBENZENE (CPFB)

	Revertants Per Plate[a]									
	TA-98		TA-1537		TA-1538		TA-100		TA-1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Negative Control[b]	36±4	48±11	6±1	8±1	14±1	24±6	112±30	138±18	23±8	17±5
2-Aminoanthracene[b] (10 µg/plate)	89±11	1413±271	14±3	282±5	22±8	2268±170	142±9	2322±30	32±6	87±1
2-Nitrofluorene[b] (10 µg/plate)	958±158	679±44			582±33	432±78				
9-Aminoacridine[b] (50 µg/plate)			267±8	64±1						
Sodium Azide[c] (10 µg/plate)							669±16	953±6	1102±20	1058±26
Sample CPFB[b]										
1.6 µl/plate	19±1[d]	42±15[d]	2±3[d]	4±2[d]	6±4[d]	16±5[d]	81±4[d]	111±4[d]	10±6[d]	11±2[d]
0.32 µl/plate	28±1[d]	47±4	5±1[d]	11±4	6±1[d]	16±2	108±8	119±12	18±3	18±8
0.064 µl/plate	48±4	50±8	7±1	6±3	14±1	14±7	143±5	130±10	22±4	13±1
0.0128 µl/plate	49±11	61±11	6±2	8±1	15±1	11±4	136±11	123±20	21±4	19±1
0.00256 µl/plate	40±0	65±3	7±1	7±1	13±5	17±4	128±6	131±5	21±6	13±7

[a] Mean of two replicate plates ± standard deviation.
[b] Dissolved/diluted in dimethylsulfoxide (DMSO).
[c] Dissolved/diluted in sterile distilled water.
[d] Toxic effect seen.

TABLE 4

CYTOTOXICITY OF CHLOROPENTAFLUOROBENZENE ON
CHO CELLS - CLONAL DETERMINATION

Concentration µg/ml	Without S9		With S9	
	Colonies/ Plate ± S.D.[a]	Treated/ Control	Colonies/ Plate ± S.D.[a]	Treated/ Control
Medium Control	219±25.7	-	61±6.7	-
0.1	217±24.1	1.06	65±6.4	1.27
0.3	208±9.0	1.02	50±11.0	0.98
1.0	219±14.5	1.07	46±7.8	0.90
3.0	209±13.9	1.02	49±7.0	0.96
10.0	211±21.6	1.03	59±3.0	1.16
30.0	200±8.4	0.98	57±2.1	1.12
100.0	116±9.5	0.58	49±13.1	0.96
300.0	-	-	39±6.2	0.76
DMSO Control	204±5.5	1.00	51±8.5	1.00

[a] Plating Density: 200 cells/plate; mean of 3 plates ± standard deviation.

TABLE 5

CHO/HGPRT GENE MUTATION ASSAY ON CHLOROPENTAFLUOROBENZENE (CPFB)
IN THE ABSENCE OF S9 METABOLIC ACTIVATION

		CYTOTOXICITY		MUTAGENICITY			
Chemical	Concentration (µg/ml)	% Cloning Efficiency	Treated/ Control	% Cloning Efficiency at Selection	Total Mutant Colonies	Mutation Frequency[a]	Statistical Significance ($p<0.01$)
Medium Control	-	79.5	-	88.0	2	1.14	No
DMSO Control	0.38%	81.0	1.00	86.0	0	<0.58	-
CPFB	10[b]	78.0	0.96	75.0	0	<0.67	No
	25[b]	80.5	0.99	71.5	2	1.54	No
	50[b]	72.0	0.89	79.0	23	14.56	Yes
	100[b]	21.0	0.26	76.5	0	<0.65	No
	250[c]	66.0	0.81	91.0	26	14.29	Yes
	500[c]	19.5	0.24	65.0	0	3.08	No
EMS	248	d	-	43.0	947	1,101.16	Yes

[a] Expressed as mutants per 10^6 clonable cells
[b] Primary stock solution = 25 mg/ml in DMSO
[c] Primary stock solution = 125 mg/ml in DMSO
[d] Toxic, colonies too small to count

TABLE 6

CHO/HGPRT GENE MUTATION ASSAY ON CHLOROPENTAFLUOROBENZENE (CPFB)
IN THE PRESENCE OF S9 METABOLIC ACTIVATION - EXPERIMENT 1

		CYTOTOXICITY		MUTAGENICITY			
Chemical	Concentration (µg/ml)	% Cloning Efficiency	Treated/ Control	% Cloning Efficiency at Selection	Total Mutant Colonies	Mutation Frequency[a]	Statistical Significance ($p<0.01$)
Medium Control	w/o S9	120.5	-	74.0	76	51.35	-
DMSO Control	0.7%	85.5	1.00	61.5	28	25.09	-
CPFB	10	75.5	0.88	53.0	55	57.65	No
	25	98.0	1.15	73.5	106	71.62	Yes
	50	86.0	1.01	61.0	72	59.02	No
	100	82.5	0.96	70.5	47	36.78	No
	250	87.0	1.02	68.0	71	52.21	No
	500	77.0	0.90	69.0	73	52.90	No
DMN	500	65.5	0.77	58.5	144	135.59	Yes

[a] Expressed as mutants per 10^6 clonable cells

TABLE 7

CHO/HGPRT GENE MUTATION ASSAY ON CHLOROPENTAFLUOROBENZENE (CPFB)
IN THE PRESENCE OF S9 METABOLIC ACTIVATION - EXPERIMENT 2

		CYTOTOXICITY		MUTAGENICITY			
Chemical	Concentration (µg/ml)	% Cloning Efficiency	Treated/ Control	% Cloning Efficiency at Selection	Total Mutant Colonies	Mutation Frequencya	Statistical Significance (p<0.01)
Medium Control	w/o S9	73.2	-	63.5	2	1.56	-
DMSO Control		79.0	1.00	55.0	2	1.82	-
CPFB	10	70.8	0.90	68.5	5	3.62	No
	25	78.2	0.92	53.5	0	<0.93	No
	50	61.6	0.78	63.0	3	2.38	No
	100	40.3	0.51	57.5	0	<0.86	No
	250	59.4	0.75	53.5	5	4.63	No
	500	37.2	0.47	66.0	6	4.54	No
DMN	500	68.1	0.86	41.0	91	110.98	Yes

[a] Expressed as mutants per 10^6 clonable cells

TABLE 8

CHO/SISTER CHROMATID EXCHANGE ASSAY ON CHLOROPENTAFLUOROBENZENE
IN THE ABSENCE OF S9 METABOLIC ACTIVATION

Chemical Concentration (µg/ml)	Control 1% DMSO	Chloropentafluorobenzene 10	30	100	300[b]	EMS 124
Mitotic Index (%)	10.5	10.5	10.4	10.4	8.3	7.0
Mean Chromosomes/Cell	20.2	20.2	20.2	20.1	20.2	20.1
± Standard Deviation	0.69	0.67	0.63	0.68	0.56	0.62
Cells Scored	50	50	50	50	50	50
Total SCE[a]	724	716	679	746	821	2384
SCE/Cell	14.5	14.3	13.6	14.9	16.4	47.7
± Standard Deviation	3.61	3.13	4.22	4.52	4.03	11.12
t-Test Score	-	0.297	1.152	0.491	2.496	20.182
p<0.05	-	No	No	No	Yes	Yes
p<0.01	-	No	No	No	Yes	Yes

[a] SCE = Sister Chromatid Exchange
[b] Some mitotic figures have chromosome aberrations.

TABLE 9

CHO/SISTER CHROMATID EXCHANGE ASSAY ON CHLOROPENTAFLUOROBENZENE
IN THE PRESENCE OF S9 METABOLIC ACTIVATION

Chemical Concentration (µg/ml)	Control 1% DMSO	Chloropentafluorobenzene 10	30	100	300	CPP 2.5
Mitotic Index (%)	11.2	11.0	11.1	10.5	10.2	9.4
Mean Chromosomes/Cell	20.3	20.2	20.2	20.3	20.3	20.3
± Standard Deviation	0.63	0.56	0.56	0.65	0.58	0.57
Cells Scored	50	50	50	50	50	50
Total SCE[a]	780	823	836	824	840	1564
SCE/Cell	15.6	16.5	16.7	16.5	16.8	31.3
± Standard Deviation	4.54	3.91	3.67	3.95	4.88	7.35
t-Test Score	-	1.068	1.339	1.063	1.280	12.916
$p<0.05$	-	No	No	No	No	Yes
$p<0.01$	-	No	No	No	No	Yes

[a] SCE = Sister Chromatid Exchange

TABLE 10

CHO/CHROMOSOME ABERRATION ASSAY ON CHLOROPENTAFLUOROBENZENE
IN THE ABSENCE OF S9 METABOLIC ACTIVATION

Chemical	Control	Chloropentafluorobenzene				EMS
Concentration (µg/ml)	1% DMSO	10	30	100	300[b]	248
Mitotic Index (%)	9.9	9.8	9.5	10.0	5.1	7.3
Chromosome Aberrations						
Marker	0	0	0	1	1	1
Break	0	0	1	0	4	12
Fragment	1	5	5	9	7	16
Gap	3	1	3	4	13	4
Chromatid Aberrations						
Interchange	0	0	0	0	1	2
Break	0	0	1	1	0	14
Fragment	0	0	0	0	0	2
Gap	2	4	4	4	2	12
Numerical Aberrations						
Pulverized	0	0	0	0	0	0
>10 Aberrations	0	0	0	0	0	0
Total Aberrations[a]	1	5	7	11	13	47
Cells Scored	100	100	100	100	100	100
Aberrations/Cell ± S.E.M.	0.01±0.01	0.05±0.03	0.07±0.03	0.11±0.04	0.13±0.04	0.47±0.08
% Cells with Aberrations	1	4	5	10	11	34
t-Test Score	-	1.440	1.744	2.754	2.918	5.864
$p<0.05$	-	No	Yes	Yes	Yes	Yes
$p<0.01$	-	No	No	Yes	Yes	Yes

[a] Exclude gaps
[b] Toxic dose, approximately 30% survival compared to negative control.

TABLE 11

CHO/CHROMOSOME ABERRATION ASSAY ON CHLOROPENTAFLUOROBENZENE
IN THE PRESENCE OF S9 METABOLIC ACTIVATION

Chemical	Control	Chloropentafluorobenzene				CPP
Concentration (μg/ml)	1% DMSO	10	30	100	300	10
Mitotic Index (%)	9.6	10.0	10.1	10.3	11.7	9.6
Chromosome Aberrations						
Marker	0	0	0	2	1	1
Break	0	0	0	0	1	8
Fragment	0	2	3	4	13	11
Gap	1	0	0	1	3	7
Chromatid Aberrations						
Interchange	0	0	0	1	1	3
Break	0	0	1	2	2	8
Fragment	0	0	0	0	4	4
Gap	5	1	3	0	4	15
Numerical Aberrations						
Pulverized	0	0	0	0	0	0
>10 Aberrations	0	0	0	0	0	0
Total Aberrations[a]	0	2	4	9	22	35
Cells Scored	100	100	100	100	100	100
Aberrations/Cell ± S.E.M.	0	0.02±0.01	0.04±0.02	0.09±0.04	0.22±0.07	0.35±0.07
% Cells with Aberrations	0	2	4	7	16	25
t-Test Score	-	1.432	2.005	2.578	3.393	5.237
$p<0.05$	-	No	Yes	Yes	Yes	Yes
$p<0.01$	-	No	No	Yes	Yes	Yes

[a] Exclude gaps

TABLE 12

PRIMARY RAT HEPATOCYTE/DNA REPAIR ASSAY ON CHLOROPENTAFLUOROBENZENE (CPFB)

Experiment 1

Chemical	Concentration µg/ml	Net Grain/ Nucleus ± S.E.[a]	% Nuclei ≥5 NG[b]	Statistical Significance (p<0.01)
Solvent Control	-	3.5±0.52	30	-
CPFB	0.1	3.2±0.44	28	No
	1.0	2.2±0.50	20	No
	10.0	1.3±0.68	24	Yes[c]
	100.0	4.4±0.41	36	No
	1000.0	4.0±0.31	32	No
DMN	30.0	8.6±0.61	84	Yes
AFB_1	0.5	9.9±0.56	92	Yes

[a] Average of 50 or 100 cells ± standard error of the mean.

[b] Percent of cells scored with greater or equal to five net nuclear grain counts.

[c] Statistically significant below the solvent control value.

TABLE 13

PRIMARY RAT HEPATOCYTE/DNA REPAIR ASSAY ON CHLOROPENTAFLUOROBENZENE (CPFB)

Experiment 2

Chemical	Concentration µg/ml	Net Grain/ Nucleus ± S.E.[a]	% Nuclei ≥5 NG[b]	Statistical Significance (p<0.01)
Solvent Control	-	-0.3±0.30	2	-
CPFB	0.1	0.8±0.26	0	Yes
	1.0	0.0±0.29	0	No
	10.0	2.3±0.40	12	Yes
	100.0	2.3±0.29	16	Yes
	1000.0	Toxic	-	-
DMN	30.0	19.3±1.06	98	Yes
AFB_1	0.5	3.6±0.27	22	Yes

[a] Average of 50 or 100 cells ± standard error of the mean.

[b] Percent of cells scored with greater or equal to five net nuclear grain counts.

TABLE 14

CYTOTOXICITY OF CHLOROPENTAFLUOROBENZENE ON
BALB/c-3T3 CELLS - MASS CULTURE DETERMINATION

Concentration µg/ml	Cells/Well[a]	Treated/Control
Medium Control	3.6×10^5	1.00
0.01	3.1×10^5	0.86
0.1	2.7×10^5	0.75
1.0	2.7×10^5	0.75
10.0	3.1×10^5	0.86
100.0	1.6×10^5	0.44
1,000.0	0.2×10^4	0.01
DMSO Control (0.5%)	2.9×10^5	0.81

[a] Plating Density: 5×10^3 cells/35 mm well; mean of duplicate wells.

TABLE 15

BALB/c-3T3 TRANSFORMATION ASSAY ON CHLOROPENTAFLUOROBENZENE

Chemical Concentration (μg/ml)	Control		Chloropentafluorobenzene					MCA
	Medium	0.5% DMSO	10	25	50	100[b]	250[b]	2.0
Total Foci/ Total Plates	13/20	7/19	12/19	12/20	14/20	10/20	8/20	47/20
Foci per Plate ± S.E.M.[a]	0.65±0.20	0.37±0.14	0.63±0.16	0.60±0.21	0.70±0.16	0.50±0.14	0.40±0.18	2.35±0.31
Total Plates with Foci/Total Plates	9/20	7/20	11/20	7/20	11/20	9/20	5/20	19/20
Ratio	0.45	0.35	0.55	0.35	0.55	0.45	0.25	0.95
T-Statistic	-	1.23	0.08	0.2	0.19	0.63	1.09	4.39
Degrees of Freedom	-	37	39	40	40	39	38	30
Statistical Significance ($p<0.05$)	-	No	No	No	No	No	No	Yes
Cells/Plate x 10^5	13.0	14.0	15.0	20.0	11.0	12.0	8.0	5.0
Treated/Control	1.00	1.08	1.15	1.54	0.85	0.92	0.62	0.38
Clones/100 Cells ± S.E.M.[a]	74±1.2	112±1.2	116±0.5	99±1.2	110±1.1	142±1.0	122±1.4	30±1.1
Treated/Control	-	1.00	1.04	0.88	1.02	1.27	1.09	0.27

[a] Standard Error of Mean.
[b] Chemical dissolved plastics, blisters formed in bottom of plates

TABLE 16

SUMMARY RESULTS OF IN VITRO SHORT TERM ASSAYS
ON CHLOROPENTAFLUOROBENZENE

Assay	-S9	+S9
Gene Mutation Assays		
Ames	-	-
CHO/HGPRT	-	-
Cytogenetic Assays		
CHO/SCE	-	-
CHO/CA	+	+
DNA Repair Assay		
Rat hepatocyte/UDS	+	N.D.[a]
Cell Transformation Assay		
BALB/c-3T3	-	N.D.[a]

[a] Not done

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